IN SITU HISTOLOGICAL EVALUATION OF ELASTASE ACTIVITY[1]

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ABSTRACT.—**A simple technique is described for demonstrating the relative extent of elastin digestion produced by various enzymes in salt-cured but otherwise intact tissue. Pieces of steer hide, or similar material, are incubated with strong solutions of enzyme at 38°C for 7 or 17 hr, washed and fixed with formalin. Frozen sections (30 to 40μ) are cut perpendicular to the skin surface, stained for elastin by the Verhoeff and by the Fullmer-Lillie methods, and mounted on slides. Microscopic examination within the zone of dense elastin is directed at determining the transition point above which the fibers have been visibly altered, but remain unchanged below. The depth of this point is expressed as percent of total depth of dense elastin to provide a numerical value for elastase activity. Such values have been obtained for papain and trypsin, as well as several other enzymes currently under investigation for use as unhairing agents.**

INTRODUCTION

Cordon (1955), Cordon *et al* (1958, 1959) and Everett and Cordon (1958) of this laboratory have recently been concerned with the development of a process for unhairing hides and skins by means of enzymes, in an attempt to replace the present chemical method which uses lime-sulfide liquors. Significant improvement in the waste disposal problem should be realized by such a process.

Unhairing requires thorough loosening of the epidermis and most of its appendages so that it can be mechanically removed, leaving the fibrous dermis or corium undamaged for conversion into leather. The uppermost (papillary) layer of the corium, commonly called the "grain" layer or *corium minor,* also contains a dense network of fine elastin fibers intermingled with the heavier collagen fibers, as shown histologically by Turley (1926) and by Roddy and O'Flaherty (1938), and stratigraphically by Mellon and Korn (1956). It has been observed histologically that some of the enzymes under study had a pronounced effect on these elastin fibers. Since the properties of the grain are of fundamental importance to the character of finished leather, it was of interest to learn more about the elastase activities of these enzymes. The first approach, described in this report, was to observe histologically the relative extent of elastolysis produced by different enzymes under standard conditions. Corresponding results with two other methods that are more quantitative will be reported elsewhere.

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Experimental Procedure

Materials. A commercial salt-cured steer hide was used as the substrate in this work. The hide was washed, drained, cut into two-inch squares and stored in a freezer until needed. All enzymes were of commercial origin and were used as received. They were prepared as 2% aqueous suspensions, containing 0.015% phenyl mercuric acetate as a bacterial inhibitor.

Enzyme treatment. A piece of prepared hide was immersed in 50 ml of enzyme solution in a foil-covered beaker, and incubated in a water bath at 38°C for 17 hr. If the effect proved to be too strong, the treatment was repeated with new materials incubated for only 7 hr. The pH was recorded at the beginning and end of incubation. Effect of pH was tested over a wide range with one preparation—from pH 4 to 10. For this purpose the hide pieces were first conditioned in water for 24 hr by frequent additions of HCl or NaOH, as required to attain the desired pH. Then the pieces were transferred to enzyme solution and incubated for 7 hr, during which time an occasional adjustment was sufficient to maintain the pH in the desired range. In tests with other preparations at higher pH, MacIlvaine buffer solution (citric acid-disodium phosphate) was substituted for water in preparing the enzyme. For our screening purpose, concentration of enzyme was always 2%, temperature was 38°C and pH was left unadjusted. The time of incubation was varied to indicate three degrees of activity: no action in 17 hr was negative, a measurable effect only after 17 hr was weak to moderate, while any activity in 7 hr was termed strong. At the end of incubation, the hide pieces were well washed in running water.

Fixation and sectioning. After washing, the hide pieces were immersed in 10% (v/v) formalin overnight, then for another whole day in a fresh solution. Cross sections were cut on a freezing microtome at 30 or 40μ and stored in 50% ethanol.

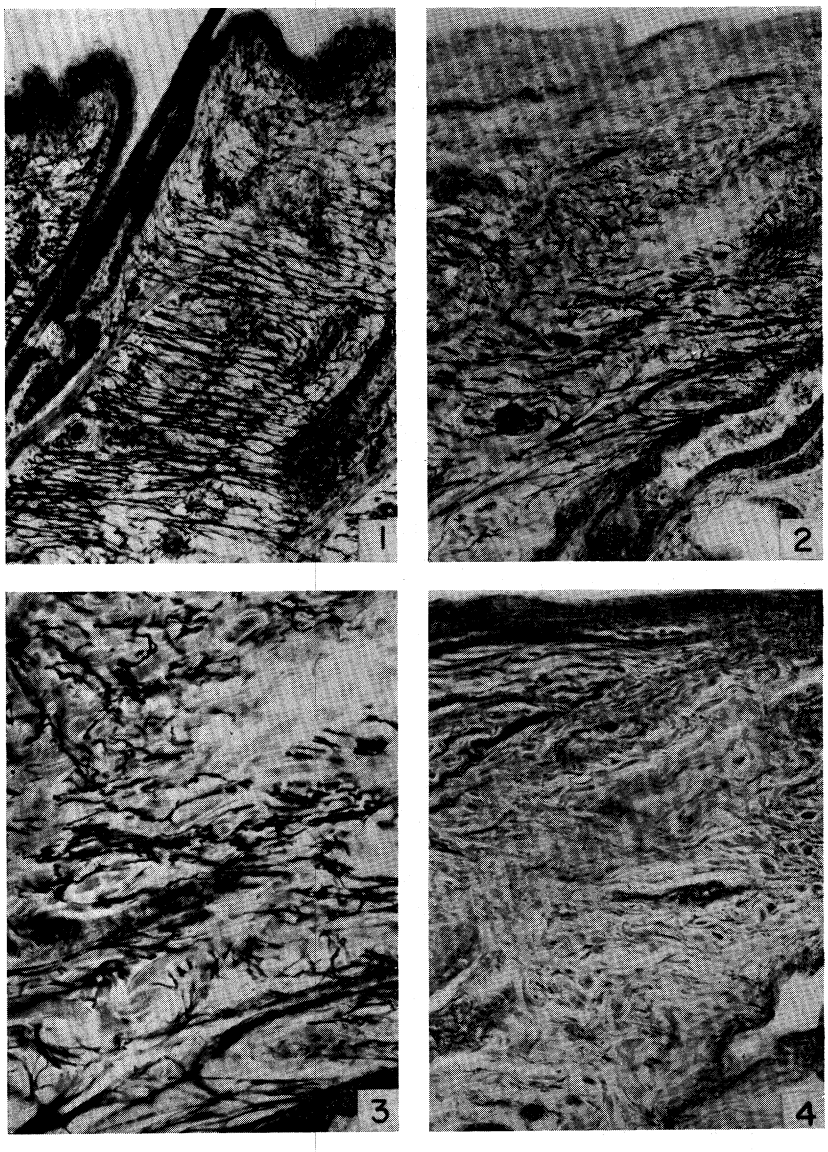
Staining. All sections were stained for elastin by several different methods, and mounted on slides for examination. Prepared Weigert's elastic tissue stain had been used quite successfully in some of our previous work, but when subsequent batches failed to give consistently good results our studies were confined to the following two methods:

1. *Verhoeff stain:* according to Mallory, as described by Lillie (1954, p. 359). Elastin is stained black by an iron-hematoxylin-iodine solution while collagen is counterstained red with eosin. This stain gave excellent results when properly differentiated and when fresh solutions were used. Even the smallest fibers were clearly visible under high power. Due to the sharp contrast in colors, visual observation was made easier and vivid color transparencies could be obtained. Also the slides are proving to be unusually permanent.

2. *Fullmer-Lillie stain:* as described by its authors (1956). Elastin is stained deep violet by orcinol-new fuchsin while other structures remain essentially unstained. Counterstaining was not necessary but, as the authors suggested, stain intensity was improved by mixing with an equal volume of Lillie's (1954, p. 81) redox buffered iron hematoxylin. This was the variant most

frequently used. Besides confirming results obtained with the Verhoeff stain, this method had the advantage that the relatively unstained background permitted better black and white photography.

Microscopic evaluation. The elastase activity of an enzyme was evaluated by microscopic study of stained sections from the treated hide piece. First



Cross-sections of steer hide, stained for elastin by the Fullmer-Lillie method, photographed with a green filter; magnification 100 × for Fig. 1, 2, and 4, 200 × for Fig. 3. Fig. 1. Untreated control. Fig. 2 and 3. Treated with papain for about 7 hr to illustrate incomplete removal of elastin. Fig. 4. Treated with papain overnight to illustrate almost complete elastin removal.

the grain layer was systematically scanned under low power (100 ×) to detect any consistent changes in the appearance of the elastin fibers, which are oriented approximately parallel to the surface in this region. The criterion of elastase activity by our method was a definite change in the morphology or continuity of the stained fibers, as demonstrated in Fig. 2, when compared with an untreated control such as that shown in Fig. 1. The next step was to estimate the relative depth to which this change had occurred. This was done by determining the position of this change in relation to fixed reference structures adjacent to this area rather than by taking a linear measurement. Thus, the layer with which we were concerned begins at the top of the corium (or junction of corium and epidermis) and ends approximately at the base of the deepest hair roots. The mid-point (50% level) runs roughly through the center of most sebaceous glands. Other points of reference are hair follicles, sweat glands, and junction of muscle with follicle. When apparent depth of change had been estimated, it was confirmed by brief examination under higher power (200 ×).

In Fig. 3, which shows the central portion of Fig. 2 under higher power, there can be seen a gradation of elastase effect, pronounced at the top but gradually disappearing at the bottom. Therefore, the zone of demarcation would be near the bottom of this field, and the evaluation of sample represented by Fig. 2 and 3 would be 50 to 60% when expressed as percent of total grain layer thickness (the lowermost portion of grain layer is not included in Fig. 2). Because of biological variability, a broader range of values is often necessary to represent results from several sections.

Fig. 4 demonstrates the extreme effect of prolonged incubation with a strong enzyme. Only a few small fragments of elastin remain, near the bottom of the picture.

Results

Enzymes from a number of sources have been evaluated in the manner described, but only some of the results are reported here. Table 1 indicates that the strongest effect was exhibited by a special preparation designated "HT Concentrate P."[4] A regular commercial batch (※4903) from the same source was more thoroughly studied in this work. By fractional adjustment of pH (between 4 and 10) it was found to have optimum activity from about 6.5 to 8.5 and moderate action over a fairly broad range. Wang *et al* (1957, 1958), using markedly different conditions, reported no activity for this and other microbial preparations. It is apparent that the optimum region for activity with papain was below pH 7, and the presence of metabisulfite as an activator did not appreciably affect the digestion of elastin. With trypsin there was some increase in activity on raising the pH closer to its usual optimum range. Crystalline trypsin, however, was devoid of elastase activity under our conditions, which makes it obvious that the elastase activity of crude trypsin is due to the presence of a nontryptic component. Microbial lipase and pectinol also appeared to be

[4] The mention of commercial products does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

TABLE 1. ELASTASE ACTIVITIES OF 2% ENZYME SOLUTIONS INCUBATED WITH STEER HIDE FOR 7 HOURS AT 38°C.

Enzyme (Source)	pH of Incubation Medium			ELASTIN Degraded, %
	Means of control	Initial	Final	
HT Concentrate P (bacterial)*	Unadjusted	6.9	6.5	75–100
HT Concentrate #4903 (bacterial)*	Adjusted†	8.5	8.5	40–60
	Unadjusted	6.8	6.3	50–75
	Adjusted	5.4	5.1	10–25
Papain, noncryst. (plant)§	Buffered‡	7.5	7.0	30–40
	Unadjusted	5.2	5.7	40–70
	" + 1% $Na_2S_2O_5$	4.9	5.4	50–60
Trypsin, noncryst. (animal)§	Unadjusted	5.9	5.6	20–40
	Buffered	7.6	7.1	30–50

* Takamine Laboratory, Clifton, N. J.
† Manual addition of NaOH or HCl.
‡ MacIlvaine buffer pH 8.
§ Nutritional Biochemicals Corp., Cleveland, Ohio.

inactive. Most of the enzymes showing unhairing activity gave intermediate values.

DISCUSSION

This method has the advantage of demonstrating an enzymic effect on elastic fibers in their anatomical environment, in contrast to procedures such as the photometric method of Sachar *et al.* (1955), which requires isolated elastin and thus necessitate more or less drastic preparative treatments. Also the method has the practical advantage of simplicity. The use of thick sections did not detract from accurate observation, but rather increased the sensitivity of evaluation by presenting more material per field without loss of resolution. Concentrated enzyme solutions were employed because the method was meant to serve as a screening test in addition to giving comparative semiquantitative results. A similar technique was proposed by Wilson and Daub (1921) using a Bismarck brown stain, although they attempted to estimate the actual amount of elastin removed. However, it is emphasized that microscopic evaluation, as defined in this method, measured only the depth of visible qualitative degradation, and did not attempt to estimate the total amount of elastin affected.

REFERENCES

CORDON, T. C. 1955. Unhairing of hides and skins by amylase preparations. J. Am. Leather Chemists Assoc., **50**, 270–4.

CORDON, T. C., JONES, H. W., CLARKE, I. D., and NAGHSKI, J. 1958. Microbial and other enzymes as depilatory agents. Applied Microbiol., **6**, 293–7.

CORDON, T. C., WINDUS, W., CLARKE, I. D., and NAGHSKI, J. 1959. A progress report on the enzyme depilation of cattle hides. J. Am. Leather Chemists Assoc., **54**, 122–39.

EVERETT, A. L., and CORDON, T. C. 1958. The use of salt to assist enzymatic unhairing of fresh hides, with histochemical observations. J. Am. Leather Chemists Assoc., **53**, 548–67.

FULLMER, H. M., and LILLIE, R. D. 1956. A selective stain for elastic tissue. Stain Techn., **31**, 27–9.

LILLIE, R. D. 1954. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., New York.

MELLON, E. F., and KORN, A. H. 1956. The network structure of elastin in the grain layer of cattle hide. J. Am. Leather Chemists Assoc., **51,** 469–79.

RODDY, W. T., and O'FLAHERTY, F. 1938. Some notes on the elastic tissue of calfskin during the manufacture of chrome leather. J. Am. Leather Chemists Assoc., **33,** 512–25.

SACHAR, L. A., WINTER, K. K., SICHER, N., and FRANKEL, S. 1955. Photometric method for estimation of elastase activity. Proc. Soc. Exp. Biol. Med., **90,** 323–6.

TURLEY, H. G. 1926. A chemico-histological study of leather manufacture. Part I. The structure and properties of freshly flayed steer skin. J. Am. Leather Chemists Assoc., **21,** 117–56.

WANG, H., WEIR, C. E., BIRKNER, M. L., and GINGER, B. 1957. The influence of enzyme tenderizers on the structure and tenderness of beef. Proc. Ninth Research Conf., Am. Meat Inst., 69–82.

WANG, H., WEIR, C. E., BIRKNER, M. L., and GINGER, B. 1958. Studies on Enzymatic tenderization of meat. III. Histological and panel analyses of enzyme preparations from three distinct sources. Food Research, **23,** 423–38.

WILSON, J. A., and DAUB, G. 1921. A critical study of bating. J. Ind. Eng. Chem., **13,** 1137–41.